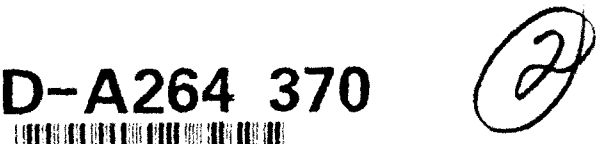

NEW APPROACHES TO HEPATITIS A VACCINE DEVELOPMENT

ANNUAL REPORT

STANLEY M. LEMON

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Stanley M. Lemon

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Hepatitis A virus (HAV) has historically been an important cause of morbibity amont U.S. soldiers in the field. Work under this contract is directed at the development of a safe, inexpensive and effective hepatitis A vaccine for use in military personnel. Two main research approaches are under investigation. First, we are characterizing neutralization escape mutants of HAV and examining synthetic oligopeptides representing the suspected surface structures of HAV. Octapeptides have been synthesized on polyethelene pins and probed with polyclonal and monoclonal antibodies in an effort to identify antigenic and potentially immunogenic sequences. We have also determined the ability of poliovirus-HAV chimeric viruses to elicit nrutralizing antibodies to HAV. The second approach involves the construction of an infectious cDNA construct derived from a virulent but cell culture-adapted variant of HAV. Genetic manipulation of such a construct will allow alternative approaches to the development of attenuated vaccine candidates. A third aim is to utilize PCR-related technology for identification of specific strains of HAV.

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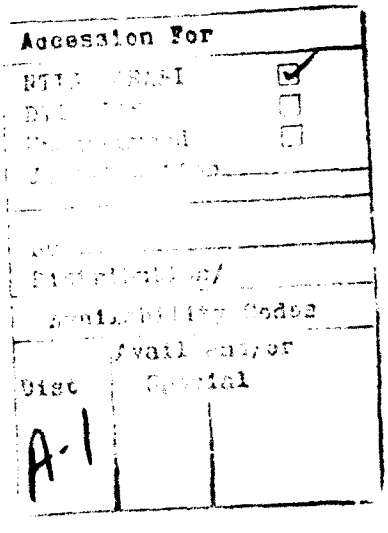
FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1985).

The investigators have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.



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ABSTRACT

Hepatitis A virus (HAV) has historically been an important cause of morbidity among U.S. soldiers in the field. Work under this contract is directed at the development of a safe, inexpensive and effective hepatitis A vaccine for use in military personnel. As in the preceding years, two main research approaches are under investigation. First, the primary goals of this contract are the characterization of neutralization epitopes on the surface of the HAV capsid and an examination of the antigenicity of synthetic oligopeptides representing the suspected surface structures of HAV. Octapeptides have been synthesized on polyethylene pins and probed with polyclonal and monoclonal antibodies in an effort to identify antigenic and potentially immunogenic sequences. These studies have recently defined a linear antigenic site on the virus capsid which is reactive with a murine neutralizing monoclonal antibody. As a subsidiary aim, we continue the investigation of poliovirus-HAV chimeric viruses which have been engineered to express potential HAV neutralization determinants. The second major approach involves basic studies related to genetic mechanisms of attenuation of HAV. This work has involved construction of a genomic length cDNA construct derived from a cell culture-adapted variant of HAV, and the characterization of mutations which enhance the growth of hepatitis A in cell culture, as these mutations are associated with attenuation of the virus. These studies now focus on mutations involving the 5' nontranslated region (5' NTR) of HAV. The 5' NTR plays a key role in determining the attenuation phenotype of the Sabin poliovirus vaccine strains and we have shown recently that mutations in the 5'NTR of HAV are critical for the enhanced growth of cell culture-adapted variants. We anticipate that these studies will lead to new approaches to the construction of genetically engineered attenuated vaccine candidates. A third aim has been the characterization of genetic diversity among wild-type HAV isolates using PCR-related technology, as this will facilitate clinical studies of HAV vaccines and future military disease surveillance activities.

INTRODUCTION

Hepatitis A as a military hazard Hepatitis A is a potentially debilitating infectious disease which may reach epidemic proportions under poor sanitary conditions, and military history records many instances in which epidemics of this disease have substantially hindered the operational effectiveness of armed forces (Bancroft and Lemon, 1984). Because hepatitis A is less prevalent in the United States than in many other countries, the prevalence of antibody to hepatitis A virus (HAV) is very low among American soldiers. Relatively recent surveys suggest that less than 15% of American soldiers have naturally acquired anti-HAV (Bancroft and Lemon, 1984), indicating that the vast majority of soldiers are susceptible to hepatitis A. In the 1990s and beyond, American forces are most likely to encounter this virus when deployed overseas to developing regions where hepatitis A may be endemic. The risk of exposure to HAV will be substantially magnified when previously existing public health facilities and sanitation practices have been disrupted by military conflict, such as occurred within the Kuwaiti Theater of Operations. The large scale mobilization of the military forces of the United States during Operations Desert Shield and Desert Storm reemphasized the risks and special problems posed by HAV to American military operations. While short-term protection against hepatitis A was provided by timely administration of immune globulin (IG) prior to deployment, supplies of IG were exhausted by the large-scale mobilization of forces. Readministration of IG is required at 6 month intervals in order to maintain continued protection. While this was not a problem due to the short duration of this conflict, reimmunization with IG may be particularly difficult to accomplish with troops engaged in action against hostile forces. There is thus an urgent need for development of a vaccine capable of providing safe, long-term, active immunity against HAV, and which would be available to the military forces of the United States at reasonable cost.

Formalin-inactivated hepatitis A vaccines Following the successful development of a prototype, formalin-inactivated HAV vaccine produced in cell culture by Army investigators (Binn et al., 1986), formalin-inactivated HAV vaccines have been developed by several commercial vaccine manufacturers (for a recent review, see Siegl and Lemon, 1990). A clinical study of the Merck inactivated HAV vaccine carried out in children in Monroe, NY, during 1991 demonstrated a high level of protection against symptomatic hepatitis A following administration of one dose of this vaccine (Werzberger et al., 1992). A similar study carried out in Thailand by U.S. Army investigators has demonstrated a similar level of efficacy after two doses of an inactivated vaccine produced by SmithKline Beecham (SKB) (Innis et al., 1992). While similar efficacy studies have not been carried out in adults, these vaccines appear to offer a level of protection that would be more than adequate for protection of military forces operating in hepatitis A endemic regions.

Several problems hinder the use of these formalin inactivated HAV vaccines by the U.S. military. Neither of these vaccines is yet licensed by the Food and Drug Administration for use within the United States. In addition, available data suggest that multiple doses of formalin inactivated HAV vaccines will be required in adults in order to elicit even moderately long-lasting protective levels of immunity. Multiple-dose schedules, with late booster doses given at least 6 months after the first immunization may prove inconvenient for use in military populations. Furthermore, the costs of these vaccines is likely to be high. This is due to the comparatively poor in vitro yields of antigen obtained with current vaccine virus strains, as well as the purification procedures required for production of an acceptable, modern vaccine. The SKB vaccine is currently sold in Switzerland at a cost of SF 39.00 (approximately US$ 27.00) per dose. Such high costs are likely to prohibit the universal use of inactivated vaccines among U.S. military forces. Because of the uncertainties concerning the future use of inactivated HAV vaccines by the

U.S. military , work under this grant focuses on efforts to develop alternative approaches to development of HAV vaccines.

Clinical trials of formalin-inactivated HAV vaccines have, however, provided useful new information concerning the level of clinical protection against disease that is afforded by minimal levels of antibody induced by immunization. Accordingly, during the past year, we have carried out extensive studies of the immune response to the Merck inactivated HAV vaccine, using sera collected from children during the Monroe study (Werzberger et al., 1992). These studies have helped to define the clinical utility of a new and very sensitive radioimmunoprecipitation method for the detection of antibodies to HAV which we have developed under support of this grant. These studies are detailed below.

Synthetic immunogens for protection against HAV In previous work, we mapped an immunodominant neutralization antigenic site on the surface of the HAV capsid by analysis of murine monoclonal antibody-resistant neutralization escape variants of HAV (Ping et al., 1988; Cox et al., 1990; Ping et al., 1992), and by characterizing the competition between such monoclonal antibodies for binding to the virus capsid (Stapleton et al., 1987). These studies indicate that the βB-βC loops of capsid proteins VP3 and VP1 contribute to an immunogenic structure on the virus surface that dominates in the human immune response (Ping et al., 1988; Day et al., 1990). Although, this site is largely conformationally defined, we reasoned that short oligopeptide sequences representative of the relevant regions of VP3 and VP1 should be both antigenic and potentially immunogenic with respect to HAV neutralizing activity. This has been shown to be the case with peptides representative of antigenic sites in other picornaviruses, including type 1 poliovirus (Chow et al., 1985), human rhinovirus 14 (Francis et al., 1987), and foot-and-mouth disease virus (FMDV) (Bittle et al., 1982). Peptide immunogens are highly stable reagents; they are potentially very inexpensive and extremely safe inasmuch as they are chemically defined. We have previously shown that only low levels of neutralizing antibody are required for protection against hepatitis A (Stapleton et al., 1985), a fact which was recently confirmed during an efficacy trial with the Merck inactivated HAV vaccine Werzberger et al., 1992) (see below). We have thus postulated that peptide immunogens may have practical application to the prevention of hepatitis A.

We have taken several experimental approaches to this problem:

(1) We continued efforts to map the neutralization epitopes of HAV by the isolation and characterization of monoclonal antibody resistant neutralization escape HAV mutants. Studies examining the neutralization resistance phenotypes of these mutants, coupled with identification of the capsid protein mutations responsible for neutralization escape, resulted in the partial mapping of epitopes recognized by most of a large panel of murine and human monoclonal antibodies (Ping and Lemon, 1992) (for details, see Annual Report No. 3).

(2) Based on the information provided in (1) above, we synthesized octapeptides representing the primary sequence of regions of the HAV capsid proteins VP3 and VP1 on polyethylene pins. We probed such peptides with monoclonal and polyclonal anti-HAV antibodies in peptide ELISAs (PEPSCAN) (Geysen et al., 1984, 1987). This approach resulted in the identification of a linear antigenic determinant within the HAV capsid structure which is recognized by a neutralizing murine monoclonal antibody (H7-C27). Three different peptides representing this linear determinant have been synthesized. During the past year, we have shown that H7-C27 specifically recognizes these peptides in solid-phase immunoassays, but that antibodies raised to the peptides in rabbits or guinea pigs do not neutralize or immunoprecipitate HAV (see below). We plan to examine the

immunogenicity of several additional peptide constructs before abandoning this approach to vaccine development.

(3) We characterized the antigenicity and immunogenicity of chimeric picornaviruses in which appropriate HAV peptide sequences have replaced residues within an antigenic loop of capsid protein VP1 of the Sabin type 1 poliovirus (Lemon et al., 1992). These antigenic chimeras were constructed using an infectious poliovirus cDNA clone which contains a mutagenesis cassette in the region encoding for VP1 (Burke et al., 1989). HAV/poliovirus chimeras have the potential of presenting HAV peptides in a conformationally constrained manner, and we have been encouraged by preliminary success with such chimeric viruses (see Progress Report Nos. 1 and 2). During the final year of this grant, we plan construction of poliovirus chimeras expressing the H7-C27 linear epitope described in (2) above.

Improved candidate attenuated HAV vaccines In addition to exploring these approaches to subunit HAV vaccines, we have continued efforts to better understand the molecular basis of the attenuation of HAV that frequently accompanies the adaptation of the virus to growth in cell culture. This work follows that supported under a previous contract with the U.S. Army Medical Research and Development Command (DAMD17-85-C-5272). Previous attempts to develop an attenuated HAV vaccine have focused on several cell culture-adapted virus strains (for a review, see Lemon, 1985; Siegl and Lemon, 1990). However, such cell culture-adapted viruses appear to replicate poorly in the primate liver, and have very poor immunogenicity in man. There is thus a need for less highly passaged cell culture-adapted variants, or for novel approaches to selecting attenuated HAV vaccine candidate strains. A better understanding of the molecular basis of attenuation for existing attenuated HAV stains and a better understanding of the mechanisms responsible for the adaptation of HAV to growth in cell culture would be very helpful to the rational development of new and potentially better vaccine candidates.

In an effort to characterize mutations which are responsible for the cell culture-adaptation and attenuation of HAV, we previously molecularly cloned and fully sequenced a cell culture-adapted HAV variant (HM175/p16 virus) which was initially isolated by Binn and coworkers at the Walter Reed Army Institute of Research. Although owl monkey challenge experiments with a passage-related virus, HM175/S30 (a neutralization escape variant) suggested that HM175/p16 might be virulent, a study carried out during the past year in collaboration with COL. James LeDuc, USAMRIID has indicated that the plaque-purified HM175/p16 virus is highly attenuated in owl monkeys (see below). This recent finding attaches additional significance to the mutations we had previously identified in the HM175/p16 virus. These mutations include only a single nonsilent mutation in protein 2C and two nonsilent mutations in 2B, proteins which have been suggested by Emerson et al. (1991) to contribute to the growth properties in cell culture and attenuation phenotype of the NIH attenuated HM175 vaccine candidate.

We have demonstrated that several mutations present within the 5' nontranslated region (5'NTR) of the attenuated HM175/p16 virus, at bases 152 and/or 203-7 and base 687, act to enhance the growth of HAV in BS-C-1 cells (see Report No. 3) (Day et al., 1992). The growth enhancing effects of these mutations are restricted to BS-C-1 cells and are not present in FRhK-4 cells which are derived from an alternative simian species. During the past year, we confirmed these findings by characterizing under one-step growth conditions the growth properties of viruses which either have or do not have these 5'NTR mutations (Day et al., 1992). We suspect that these mutations alter the affinity of the 5'NTR RNA for specific cellular proteins which play a role in viral replication, possibly by supporting or repressing viral translation. These hypothetical alterations in the affinity of the 5'NTR RNA for cellular proteins could be due either to subtle changes in the

secondary or tertiary structure of the RNA, or to changes in the primary structure (RNA sequence) of a protein binding site. The fact that similar 5'NTR mutations have been shown to be critical for the attenuation of the Sabin oral poliovirus vaccine strains suggests that these 5'NTR mutations may play a role in the attenuation of HM175/p16 virus. These findings have thus led us to initiate a detailed analysis of structure-function relationships within the 5'NTR of HAV, including characterization of cellular proteins which specifically bind to this region of the viral genome.

In previous work, we had developed a model of the secondary structure of the 5'NTR of HAV and identified elements within the 5'NTR which are essential for translation in vitro (Brown et al., 1991). More recent studies examining the translation of bicistronic RNA transcripts in rabbit reticulocyte lysates have demonstrated the existence of an "internal ribosomal entry site" (IRES, otherwise known as a ribosomal landing pad) located downstream of nt 152. These studies have shown that HAV replication involves a translational strategy similar to that of other picornaviruses. However, studies carried out during the past year have shown unequivocally that the translational activity of the IRES of HAV is very low in vivo, even within permissive BS-C-1 cells. These studies suggest that low level IRES activity may contribute to the slow and inefficient replication cycle of HAV. Mutations within the 5'NTR of the attenuated HM175/p16 virus which result in adaptation of the virus to growth in BS-C-1 cells are located either within the IRES of HAV or close to its 5' limit. During the past year, we have also made considerable progress in identifying specific cellular proteins which bind to the 5'NTR of HAV. At least 5 such proteins have been identified by a combination of gel mobility shift and UV cross-linking studies. We are particularly intrigued by the fact that cellular proteins which bind to the 5'NTR appear to differ between cell types. Studies are in progress to better define the nature of these proteins, and to determine whether the affinity of the viral RNA for these proteins is altered by mutations which are present in the attenuated HM175/p16 virus. These recent studies are described in greater detail in the body of the report.

We have shown that the HAV 5' NTR structure is very similar to that of the murine cardioviruses. Because cardioviruses with deletion mutations within the poly-C tract of the cardioviral 5' NTR may be highly attenuated in vivo, yet grow well in cell culture, (Duke et al., 1990), we suspected that HAV mutants with deletions within the first pyrimidine-rich tract (bases 95-152) might have interesting attenuation properties. We have generated several viral mutants with deletions with this region, and are currently assessing their growth properties in cell culture.

Genetic variation among HAV strains Finally, we reported in years 1 and 2 the development of a simplified polymerase chain reaction (PCR)-based method for analysis of the nucleotide sequence of wild-type HAV isolates, and its application to the characterization of wild-type HAV strains (Jansen et al., 1990). We subsequently collaborated with Dr. Betty Jo Robertson of the Viral Hepatitis Branch of the Centers for Disease Control, Atlanta, GA, and Yasuo Moritsugu of the National Institute of Health, Tokyo, Japan, in evaluating the genotype of over 150 unique HAV isolates and antigen-positive fecal samples collected from all regions of the world (see Report No. 3). These strains comprise the vast majority of all HAV strains available worldwide. This effort provided new information concerning the genetic diversity of HAV and the molecular epidemiology of the virus (Robertson et al., 1992), which will be useful to future military and civilian disease control efforts. Because specific goals in this area had been reached, no further work was carried out in this area during the past year.

RESEARCH PROGRESS

1. Analysis of synthetic HAV peptides

As described in previous reports and in our initial proposal, our approach to mapping potentially antigenic sites of HAV has included the synthesis of nested octapeptides on polyethylene pins. These peptides, overlapping each other by 7 residues, have been probed in enzyme-linked immunosorbent assays (ELISA) which assess the binding of immunoglobulins to specific peptide-bearing pins (PEPSCAN) (Geysen et al., 1984, 1987) (see previous Annual Reports). In previous years, we screened pin-based octapeptides representative of the putative B-C loops of VP3 (residues 3-050 - 3-091*) and VP1 (residues 1-080 - 1-130) of HAV against the panel of neutralizing anti-HAV monoclonal antibodies described above. No reactivity was demonstrated with these monoclonal antibodies, even though analysis of escape mutants (see Report No. 3) indicated that some of these antibodies recognize epitopes which are comprised, in part, of amino acid residues 3-070, 3-074 or 1-102. Thus, these epitopes are highly conformational and do not contain linear, antigenic peptides.

As described in Report No. 3, we extended the PEPSCAN analysis to include the region from 1-131 to 1-300, as we demonstrated that this region contains two additional domains involved in epitopes recognized by neutralizing monoclonal antibodies (Ping et al., 1992) (see Report No. 3). We found that the murine monoclonal H7-C27 specifically and reproducibly binds a set of nested octapeptides representing a complex linear antigenic site comprising residues between Glu-256 and Asp-283 of VP1 (Figures 1 and 2) (see Report No. 3). These data represent the first evidence for a linear antigenic site in the HAV capsid, and reasonably raised our expectations that synthetic peptides representing this domain may be immunogenic and capable of eliciting neutralizing antibody responses.

As determined by the octapeptide ELISA assays (Figure 2), the H7-C27 epitope appears to be a complex structure containing two discontinuous segments of VP1 which are each capable of functioning as linear antigenic determinants. The amino terminal element (with a core activity represented by the sequence "-Phe-Pro-Arg-Ala-Pro-Leu-Asn-", residues 1-259 through 1-265) has greater antigenic activity than the carboxy terminal element ("-Ser-Met-Met-Ser-Arg-Ile-Aia-Ala-", residues 1-274 through 1-281). To determine which residues within the amino terminal element might play critical roles in binding of the H7-C27 antibody, we synthesized additional octapeptides representing residues 1-258 through 1-265, and 1-260 through 1-267, in which the Pro-260 and/or Pro-265 residues were replaced by glycines, or Tyr-258 replaced with a threonine residue, and determined the ability of these peptides to bind H7-C27 in peptide ELISA assays (Table 1). This partial "replacement synthesis" demonstrated that Pro-263 plays a critical role in the formation of the H7-C27 epitope, as replacement of Pro-263 with glycine completely abolished antigenic activity. In contrast, solitary replacement of Pro-260 with glycine did not reduce antigenicity of the octapeptide, while replacement of Tyr-258 with threonine resulted in a minimal-moderate reduction in antigenicity. This latter finding suggests that Tyr-258 may play a minor role in determining antibody binding, a fact consistent with the modest antigenic activity of the peptide "Glu-Phe-Tyr-Phe-Pro-Arg-Ala-Pro" (residues 1-256 to 1-263) (Figure 2).

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*In this report, specific capsid protein amino acid residues are designated by a 4 digit code ("y-xxx"), in which "y" represents the specific capsid protein (VP1, VP2, or VP3) and "xxx" the residue number from the proposed amino terminus (Cohen et al., 1987).

It is interesting that H7-C27 is the only monoclonal antibody which recognizes this linear epitope. Our previous studies of neutralization escape mutants of HAV had suggested that H7-C27 recognizes an epitope which is distinctly different from that bound by other murine antibodies (Ping et al., 1992) (see Report No. 3). We found that each of four unique neutralization escape variants selected under pressure of H7-C27 had amino acid substitutions at residue 1-221, located approximately 34 residues upstream of the linear H7-C27 epitope identified by PEPSCAN. The PEPSCAN data thus suggest that the 1-221 escape mutations which are responsible for resistance against H7-C27 mediated neutralization may not be located within the primary antibody binding site, but instead confer resistance by altering the conformation of a VP1 loop containing residues 1-256 through 1-283. This is very unusual for neutralization escape mutations identified in other picornaviruses, which almost always appear to be located within the primary antibody binding site on the virus surface. However, Parry et al. (1991) recently described a foot-and-mouth disease virus (FMDV) mutant in which neutralization resistance was due to the conformational effects of a mutation occurring outside of the antibody binding site. If correct, this hypothesis would imply that the domain between residues 1-256 and 1-283, while containing linear antigenic determinants, has conformational attributes that may be important to neutralization. Alternatively, it may be that H7-C27 recognizes two adjacent VP1 loops on the surface of the virus, one of which contains residue 1-221 and which is strictly conformational, and one which contains residues 1-256 through 1-283.

In general, crystallographic studies have shown that the carboxy termini of the capsid proteins of picornaviruses are located on the surface of the capsid, while the amino termini are usually buried within the capsid. It was surprising, therefore, that the linear epitope recognized by H7-C27 terminated at residue 1-283 and did not extend to the carboxy terminus of VP1 proposed by Cohen et al. (Cohen et al., 1987). The most likely explanation for this discrepancy is that the correct carboxy terminus of VP1 is in fact located in the region of residue 1-283. In support of this argument, Stapleton and colleagues have shown recently that the native VP1 molecule present in infectious HAV is somewhat smaller than a protein expressed by a recombinant vaccinia virus and comprising the 300 amino acids proposed originally to represent VP1 (J.T. Stapleton, personal communication).

To determine whether the H7-C27 linear epitope might be a useful antigen, we synthesized large amounts of three peptides which represent segments of this putative antigenic site (Table 2). Peptide 4918 and 4919 represent the amino terminal element of the epitope (residues 1-256 to 1-268), with carboxy- and amino-terminal (respectively) Gly-Gly spacers and Cys residues for coupling to carrier. Peptide 4920 represents residues 1-256 to 1-283, with a carboxy terminal Cys residue for coupling to carrier. In preliminary studies, solid-phase peptide ELISA assays demonstrated that H7-C27 (but not another monoclonal antibody, K2-4F2) specifically recognizes peptides 4918 and 4919 (Figure 3), but not peptide 4920. These results confirmed both the validity of the peptide synthesis as well as the fact that the peptide sequence spanning residues 1-256 to 1-268 is located on the surface of HAV and contribute to immunogenic epitopes. The lack of antigenic activity of peptide 4920 is unexplained, but may be due to the inclusion of hydrophobic residues which do not participate in the epitope (see below).

Each of the three peptides was conjugated to keyhole limpet hemocyanin (KLH) through amino- or carboxy-terminal Cys residues (see Table 2). In collaboration with Dr. John Cullen of the College of Veterinary Medicine of North Carolina State University, pairs of guinea pigs and rabbits were immunized with each of the peptide-KLH conjugates. These peptide antisera have been characterized by PEPSCAN to determine the specific anti-peptide activity of each (Table 3). In addition, the peptide antisera were also tested for the ability to neutralize or immunoprecipitate (see below) infectious HAV. While good

anti-peptide activity was demonstrated by PEPSCAN (Table 3), confirming again the correctness of the peptide synthesis, none of these antisera contained detectable neutralizing or immunoprecipitating activity directed against the native virus.

The discontinuous, complex nature of the H7-C27 epitope identified by PEPSCAN (Figure 2, see above) suggests that the VP1 loop containing residues 1-256 through 1-283 may assume a dominant conformation in which part or all of the relatively hydrophobic sequence "-Ser-Asn-Ala-Met-Leu-Ser-Thr-Glu-" (residues 1-266 through 1-273) may be buried and not available for antibody binding. We are thus planning the synthesis of a series of peptides in which these residues ("-Met-Leu-", "-Ala-Met-Leu-Ser-", and "-Ser-Asn-Ala-Met-Leu-Ser-Thr-Glu-") are deleted in a nested fashion from the 1-256 to 1-284 domain. These peptides will be coupled to KLH through amino terminal "Cys-Gly-Gly-" residues, so that the free carboxy terminus of the peptide may mimic the putative free end of VP1. Peptides will be tested for antigenicity and immunogenicity, as described above. In addition, we plan to insert peptide sequences representing the amino element of the epitope, as well as the complete epitope (residues 1-256 through 1-284) into antigenic site 1 within the VP1 protein of Sabin type 1 poliovirus (see previous Reports). We will assess the antigenicity and immunogenicity of the resulting antigenic viral chimeras, as described previously (Lemon et al., 1992). These studies should allow us to determine whether the antigenicity of the HAV peptide sequence is favorably constrained by expression on the surface of an antigenically unrelated picornavirus.

2. Development of a radioimmunoprecipitation assay for detection of antibodies to HAV

We demonstrated previously that soldiers who had received putatively protective doses of immune globulin (IG) did not have levels of antibodies to the virus that were detectable in solid-phase competitive inhibition immunoassays (Stapleton et al., 1985). These early data indicated that the minimal protective level of anti-HAV was very low. This was confirmed in recent studies examining the protective efficacy of inactivated HAV vaccines (Werzberger et al., 1992). Virus neutralization assays, such as the radioimmunofocus inhibition assay developed at WRAIR (Lemon and Binn, 1983), are approximately 10-100 fold more sensitive than commercial immunoassays for detection of anti-HAV. Neutralization assays are capable of detecting low levels of protective antibodies in passively as well as actively immunized individuals. However, neutralization assays are time consuming, complex, labor-intensive, and very expensive. In order to develop a simple and rapid procedure for the sensitive detection of low levels of anti-HAV, we have devised a test procedure which is based on the immunoprecipitation of metabolically labeled radioactive HAV.

HAV (HM175/18f strain) was labelled by a process involving high-multiplicity infection of BS-C-1 cells in the presence of [^{3}H]-uridine. In general, this is a very inefficient process, because HAV infection does not induce host cell metabolic shutdown. Surprisingly, the addition of actinomycin-D to the culture medium did not enhance the incorporation of [^{3}H]-uridine into the virus, and in fact resulted in a significant reduction of the virus yield. While further study is required, this finding suggests that ongoing synthesis of some cellular proteins may be absolutely required for efficient HAV replication. (We also considered the use of guanidine, because Ehrenfeld and coworkers have reported that HAV RNA synthesis occurs rapidly following release of infected cells from guanidine blockade. However, we were not able to confirm that guanidine inhibits replication of the virus.) Labelled virus was recovered from infected cell culture lysates, partially purified by centrifugation through a rate-zonal sucrose gradient, and concentrated by centrifugation dialysis prior to use.

For immunoprecipitation assays, labelled virus (5-10 x 10^2 cpm in 10 ul) was mixed with 90 ul aliquots of ten-fold serum dilutions in NTE buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.9, 5 mM EDTA, and 0 1% NP-40) and incubated at 4^o C overnight. Following the addition of 50 ul of washed protein A-bearing staphylococcal cells (Pansorbin, Calbiochem), mixtures were held at room temperature for 90 min, and briefly centrifuged in a table top microcentrifuge. Pellets and supernatant fractions were counted separately in a B-scintillation counter. The endpoint antibody titer was calculated as that dilution providing greater than 30% precipitation of the labelled virus. Once labeled virus has been prepared, the assay is relatively simple to carry out and is amenable to the analysis of large numbers of serum specimens.

Results to date indicate that this immunoprecipitation assay is significantly more sensitive than the radioimmunofocus inhibition assay for detection of antibody in recipients of inactivated HAV vaccines. In general, most antibody-negative preimmunization sera do not precipitate more than 25% of labelled virus, while sera collected from children one month after a single dose of the Merck inactivated HAV vaccine precipitate a substantially greater proportion of the labelled virus (Figure 4). Detection of antibody appears optimal with substantial pre-dilution of the serum sample (about 1:80). Table 4 shows results of a study in which antibody to HAV was detected by radioimmunoprecipitation as well as radioimmunofocus inhibition assays, in children who had received two doses of the Merck HAV vaccine (at 0 and 6 months). These studies were done in collaboration with Dr. John Lewis of the Merck Research Laboratories. The immunoprecipitation assay should prove very useful for evaluating the immune response to new hepatitis A vaccines. For reasons that remain obscure and are still under investigation, however, the immunoprecipitation assay appears to be less sensitive than the neutralization assay in recipients of immune serum globulin (data not shown). This surprising finding suggests that there may be qualitative as well as quantitative differences in circulating anti-HAV following passive vs. active immunization. We are currently extending these studies to larger patient cohorts, including children who participated in the Monroe trial of the Merck vaccine.

3. Attenuation phenotype of the HM175/p16 virus

In collaborative studies with COL. J. LeDuc and MAJ K. Taylor of USAMRIID, we have characterized the attenuation phenotype of a clonally-isolated, low-passage, cell culture-adapted virus (HM175/p16) derived from the HM175 strain of HAV (see previous Annual Reports). Because we have previously determined the complete nucleotide sequence of HM175/p16 virus (Jansen et al., 1988), recognition of its attenuation phenotype adds substantially to the body of available knowledge concerning mutations associated with the attenuation of HAV.

In this collaborative study, we assessed the attenuation/virulence phenotype of HM175/p16 virus in 4 anti-HAV negative Aotus nancymai monkeys. Baseline serum chemistries (serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transpeptidase (GGT) and total bilirubin) were monitored in each animal over a 10 month period prior to BL-2+ containment (a total of 10 observations). Chemistries were measured with the Ektachem Clinical Chemistry Slide system (Kodak, Rochester, NY). Following a 14 day BL-2+ acclimatization period, an open, 1.5-2.0 cm wedge biopsy of the liver was obtained from each animal under intramuscular Telazol and isoflurane gas anesthesia. Following the biopsy procedure, throat swabs and fecal collections were taken 2-3 times each week for virus isolation, along with twice weekly blood samplings for monitoring of liver function tests. Forty-four days after the biopsy, each monkey was inoculated intravenously via the lateral saphenous vein with 1.0 mL of the HM175/p16 virus suspension ($10^{4.5}$ rfu). Throat swabs and blood samples were collected twice weekly after inoculation, while fecal samples were collected approximately

three times each week for the 7 weeks following inoculation. Additional sera were collected weekly through day 96 after inoculation, and on days 307 and 350. A second open wedge biopsy of the liver was obtained from animals 769 and 778 twenty-one days after inoculation of virus, when liver enzyme (ALT, AST) elevations in 769 suggested acute hepatocellular injury. Animals 1017 and 1022 were biopsied 37 days after inoculation, to confirm the absence of hepatic inflammation.

During the 10 month period of baseline observation, serum ALT values in the four owl monkeys ranged from 49.5 to 68.3 units/L, and AST values from 99.8 to 161.9 units/L. Significant elevations of these serum enzymes (greater than 2.5 fold above the baseline mean value) occurred in only one animal (769) following intravenous inoculation with the HM175/p16 virus (Figure 5). This animal had a baseline mean ALT of 68.3 units/L, and developed serum ALT levels of 202 and 221 (reflecting 3.0- and 3.2-fold increases above the baseline mean), 15 and 26 days following virus inoculation. AST levels in animal 769 also peaked 15 days after inoculation, at 3.1-fold above the mean baseline value (data not shown). Only minimal changes in GGT activity were observed in all 4 animals, and these appeared more likely to be related to the biopsy procedures than to virus infection. A slight upward trend in serum GGT activities was noted following the initial liver biopsy (Figure 5), but the GGT levels stabilized within the normal range reported for Aotus nancymai. Maximum serum GGT levels were only 1.7- to 1.9-fold greater than the mean baseline values (which ranged from 18.0 to 22.0), and in two animals actually peaked prior to inoculation with virus. The total bilirubin values for all of the animals remained well within normal ranges established for Aotus. Thus, only animal 769 demonstrated biochemical changes consistent with acute hepatitis A following intravenous inoculation with HM175/p16 virus; aminotransferase activities were only minimally elevated in this animal.

Liver biopsies were examined by Dr. L. Asher of WRAIR. The initial biopsies from all 4 animals showed normal histology, and normal ultrastructure by electron microscopy. Liver biopsies taken 21 days after inoculation from monkeys 769 and 778 showed minimal non-specific changes: swelling of hepatocytes and increased eosinophilia and granularity of the cytoplasm with focal disruption of the liver cell plates. Biopsies taken 37 days after inoculation from monkeys 1017 and 1022 (day 37) were normal. Inflammation and necrosis were not present in any of the biopsies taken after inoculation. The only abnormalities noted by electron microscopy were in the second biopsy from 769. This biopsy specimen revealed degenerative changes in the cytoplasm of hepatocytes, including swollen mitochondria, dilated endoplasmic reticulum and disrupted cellular membranes. In addition, 18-20 nm single particles were present in small vesicles within a cell of unidentified type located in the sinusoidal space. The nature of these particles and their potential relationship to the virus infection was not established. Viral antigen was not detected in tissue sections by immunofluorescence.

Only 3 of the 4 animals developed antibodies to HAV following inoculation with HM175/p16 virus. The earliest and strongest antibody response occurred in animal 769, which developed antibodies to HAV that were detectable in a competitive-inhibition radioimmunoassay by 40 days after inoculation (Figure 6). Sera collected from this animal 60 or more days after inoculation competed strongly (generally >85%) with radiolabeled human polyclonal IgG for binding to HAV in the radioimmunoassay. Antibody was not detected in animal 1017 until 68 days after inoculation, and only competed weakly (up to 66%) with labelled antibodies in the radioimmunoassay even at 96 days following inoculation (postinfection chimpanzee serum employed as a control in these assays generally blocked the binding of over 90% of labelled human polyclonal antibody) (data not shown). These "early" seroconversions (less than 96 days after inoculation) were confirmed in animals 769 and 1017 by the much more sensitive virus neutralization and

virus immunoprecipitation assays. Both of these animals had HAV neutralizing antibody titers in excess of 1:128 by 96 days after inoculation. In contrast, animals 778 and 1022 remained seronegative even when tested by these assays 96 days following inoculation.

Antibody was not noted in the third animal (1022) until 307 days following inoculation, when all four animals were rebled and tested by a commercial immunoassay (HAVAB). Only animal 778 remained seronegative at this point in time. Further testing on sera collected from the animals 350 days after inoculation confirmed a serologic response to HAV in animal 1022. Antibodies in serum taken from this animal on day 350 competed weakly with labelled human polyclonal antibody in the radioimmunoassay (only 44%), but precipitated over 50% of tritiated virus particles when tested at serum dilutions of 1:8, 1:80, and 1:800. The interval between inoculation of HM175/p16 virus and the first appearance of antibodies to HAV was substantially longer than that we have observed in earlier studies of owl monkeys inoculated with virulent hepatitis A viruses carried out in collaboration with USAMRIID and WRAIR investigators (Table 5).

Because HM175/p16 virus is well adapted to growth in cell culture, it was possible to monitor virologic events in infected monkeys using radioimmunofocus assays which provided a quantitative measure of the titer of virus present. Viremia was demonstrated only in animal 769, in which virus was present continuously in the serum from day 8 to day 33 following inoculation, peaking at 2.7×10^3 rfu/ml on day 33 (Figure 6). Fecal samples collected from the 2 animals which developed antibodies to HAV within 96 days of inoculation were similarly tested by radioimmunofocus assay for the presence of infectious virus. Infectious virus was identified only in the feces of animal 769 (Figure 6). This animal shed infectious virus from 12 to at least 44 days (the last day tested) following virus inoculation. Peak fecal shedding of virus (1.3×10^5 rfu/gm feces) occurred 30 days after inoculation. Virus was not recovered from fecal samples collected from animal 1017 up to 44 days after inoculation, although this animal also developed anti-HAV antibodies within 68 days of inoculation. None of the fecal samples collected from 769 or 1017 generated a reproducible positive result in a radioimmunoassay for detection of HAV antigen (data not shown). This reflects the low level of fecal shedding even by animal 769. Virus was not recovered in cultures inoculated with throat swabs taken from any animal.

Prior to this study, we considered it likely that the HM175/p16 virus would be shown to be virulent and capable of causing acute hepatocellular disease in Aotus monkeys. The evidence favoring this hypothesis included the occurrence of acute hepatitis in 6 of 6 owl monkeys inoculated intravenously with the passage-related HM175/S18 virus (Table 5). HM175/S18 virus shares a common passage history with HM175/p16 through the first 11 passages in cell culture, and had undergone a total of 22 cell culture passages prior to inoculation into primates (6 more cell culture passages than HM175/p16 virus). As with HM175/p16, HM175/S18 virus had been clonally isolated from agarose overlying radioimmunofoci (Lemon et al., 1990). However, HM175/S18 virus was selected for its ability to escape neutralization mediated by the monoclonal antibody K2-4F2, which is due to an aspartic acid to histidine substitution at residue 70 of the capsid protein VP3. A previous study in owl monkeys demonstrated that this escape mutant had only a limited ability to replicate in monkeys, but reversion of the His-70 mutation rapidly occurred (or was selected for) in all 6 inoculated animals. Replication of this revertant virus resulted in acute hepatocellular damage with substantial ALT elevations, histologic evidence of inflammation, and early seroconversion to anti-HAV positivity. The only evidence for attenuation of the HM175/S18 virus that could be related to its cell culture passage history was that the shedding of viral antigen in feces of infected owl monkeys appeared to be significantly reduced compared with that noted in an earlier study involving inoculation of owl monkeys with a 0.2% suspension of human feces containing wild-type HM175 virus. When fecal suspensions were tested by radioimmunoassay for HAV antigen, the mean

maximum signal-to-noise (S/N) ratio was 14.6 in animals infected with HM175/S18 virus, compared with 73.2 in animals infected with wild-type virus (Table 5).

The genome of HM175/p16 virus contains a total of 19 mutations from the reported sequence of the wild-type virus genome (Jansen et al., 1988). Only 8 of these mutations result in amino acid substitutions within the HAV polyprotein, while 6 mutations are located within nontranslated regions of the genome. Current evidence suggests that a relatively small number of mutations in the P2 (proteins 2B and 2C) and 5' nontranslated regions (5'NTR) of the genome are responsible for cell culture-adapted phenotypes of the virus (Emerson et al., 1992; Day et al., 1992). Less is known about mutations in the viral genome which result in attenuation of the hepatovirulence of HAV. Although these two virus phenotypes are related, it is clear that not all cell culture-adapted viruses are attenuated (Table 3). Cohen et al. (1989) inoculated marmosets with viruses rescued from chimeric infectious cDNA clones derived from wild-type and attenuated (HAV/7 virus) variants of the HM175 strain of HAV. The results of these studies suggested that partial (but incomplete) attenuation of HAV/7 virus for marmosets could be attributed to mutations within the P2 and P3 regions of the HAV genome). Although independently adapted to growth in cell culture, the sequence of HAV/7 shares a number of mutations from the wild-type genome in common with HM175/p16 virus within the 5' nontranslated region (5'NTR), and the VP2, 2B and $3D_{pol}$ proteins (Jansen et al., 1988). However, further studies will be required in order to learn whether or not the attenuation phenotypes of HM175/p16 and HAV/7 derive from a common set of mutations that are present in both virus variants. It is not clear whether the minimal disease and more active replication of HAV observed in animal 769 represented partial reversion of the attenuation phenotype of HM175/p16 virus. It will be important in subsequent studies to assess the virulence of virus shed by this monkey.

4. Role of 5' NTR mutations in adaptation of HAV to growth in cell culture

Passage of human HAV in cell culture results in progressive increases in the efficiency with which the virus replicates in cell culture and, as discussed above, may also lead to attenuation of the virus. The presence of identical mutations within the 5'NTRs of the attenuated HM175/p16 and HM175/p35 (HAV/7) viruses has suggested that this region of the genome may play a role in determining the change in virus host range displayed by these two independent isolates (Jansen et al., 1988). We therefore constructed chimeric infectious cDNA clones in which regions of the 5'NTR of HM175/p35 (HAV/7) virus were replaced with cDNA from either wild-type virus (HM175/wt) or HM175/p16 virus. These studies were described in part in last year's report (see Report No. 3). Substitution of the complete 5'NTR of HAV/7 with the 5'NTR of HM175/wt resulted in virus with very small replication foci in continuous monkey kidney (BS-C-1) cells, indicating that 5'NTR mutations in HAV/7 virus are important for growth in these cells (Day et al., 1992). However, replacement of the HAV/7 5'NTR with the 5'NTR sequence of HM175/p16 resulted in a virus which retained the large focus phenotype of HAV/7 virus, indicating that mutations present in HM175/p16 could substitute for those present in HAV/7. The growth properties of other viruses having chimeric (HM175/p16 - HAV/7) 5'NTR sequences indicated that mutations at bases 152 and/or 203-7 are required for efficient replication in BS-C-1 cells, while a mutation at base 687 of HM175/p16 has a minor role in enhancing growth. In sharp contrast to these results, the mutations at 152 and 203-7 did not enhance growth of the virus in FRhK-4 cells (Day et al., 1992). During the past year, we confirmed the importance of the mutations at 152 and/or 203-7 by studying the growth of selected viruses with chimeric 5'NTRs under one-step growth conditions in BS-C-1 cells (Figure 7). These one-step growth curves were carried out in 24 well plates, with virus yields assessed by radioimmunofocus assays (Day et al., 1992). While somewhat tedious, this approach represents a highly quantitative method

for determination of the replication competence of hepatitis A viruses in specific cell types, and should prove useful for future studies of this type.

This series of experiments has shown that mutations at bases 152 and/or 203-7 of the 5'NTR enhance the replication of HAV in cell culture, but do so in a highly host cell-specific fashion. Because recent data indicates that HM175/p16, like HAV/7 virus, is substantially attenuated in otherwise susceptible primates (see above), these data also raise the distinct possibility that these 5'NTR mutations may contribute to the attenuation phenotype of HM175/p16 virus.

5. Role of the 5' NTR of HAV in controlling translation in vitro

The genomic RNAs of picornaviruses such as hepatitis A virus lack a 5'm7G cap, and appear to be translated by a unique mechanism involving internal entry of the 40S ribosomal subunit many hundreds of bases downstream of the 5' terminus of the RNA. Translational control thus represents an important aspect of the replication cycle of picornaviruses, and appears to be highly dependent upon the primary and secondary structure of the 5'NTR. Translation of poliovirus RNA is enhanced by the shut-down of capped cellular RNA translation that is induced by the poliovirus 2A protein. HAV lacks a comparable 2A activity, and this has been suggested to be one possible explanation for the generally slow and noncytolytic replication of the virus (Ticehurst et al., 1989).

As described in Annual Report No. 2, we developed a model of the secondary structure of the 5'NTR of HAV which was based on a phylogenetic analysis of sequence covariance within the 5'NTRs of 9 unique human and simian HAV strains (see also Brown et al., 1991). We manually identified covariant nucleotide substitutions predictive of conserved helical structures, and utilized this information to constrain the folding of the 5'NTR in thermodynamic predictions of secondary structure made by the RNAFOLD (Wisconsin Software Package) and STAR programs. The resulting model was subsequently tested and refined by nuclease digestion experiments in which we determined the effects of single- and double-strand specific ribonucleases on synthetic RNA in the region between bases 280-735. This work led us to propose the structure shown in Annual Report No. 2 for the 5'NTR of HAV (see also, Brown et al., 1991).

In Report No. 3, we described preliminary studies of the translational activities of bicistronic RNA transcripts in which translation of the second (downstream) cistron is under control of the 5'NTR of HAV (HM175/p16 sequence). These studies have shown that the 5'NTR of HAV, like the 5'NTRs of other picornaviruses, contains an internal ribosomal entry site (IRES) which is responsible for the initiation of viral translation at an internal location, many hundreds of bases from the 5' end of the RNA (see Annual Report No. 2). Bicistronic constructions were made as a single transcriptional unit under the control of the T7 promoter in the vector pGEM3/zf$^-$. The T7 terminator sequence of pGEMEX-2 (Promega) was placed downstream of the second reporter gene (the HAV capsid precursor VP0-ΔVP3) to facilitate eventual use of these plasmids in the BT7-6 cellular expression system (see below). The upstream reporter gene encoded the small form of hepatitis delta virus antigen ($p24^6$). We determined the translational activity of these bicistronic RNAs in rabbit reticulocyte lysates (Promega) which were programmed with synthetic RNAs transcribed under direction of T7 polymerase. As described in Report No. 3, when the complete HAV 5'NTR and P1 coding region were placed downstream of the $p24^6$ gene, there was abundant translation of the HAV polyprotein. However, expression of the HAV polyprotein was dependent upon the presence of an intact 5'NTR, confirming the existence of an IRES. HAV translation was markedly reduced when bases 1-354 of the NTR were removed, indicating that the 5' limit of the IRES extends upstream of base 355. In the past year, a number of additional 5' deletion mutants have been

generated and tested in this system. Analysis of these additional constructions suggests that the 5' limit of the IRES extends into domain III (see Report No. 2) of the 5'NTR: 5' deletions up to nt 151 did not reduce IRES activity, whereas deletion of nts 1-253 significantly reduced translation in vitro. Other constructions were made which have internal deletions near the 3' end of the 5'NTR. The HAV translational activity of these transcripts was also very low. These data thus define the existence of an IRES which is located between nts 152 and the first initiator AUG at nt 735.

6. HAV translational control in vivo

Since important differences have been found between translation of poliovirus RNA in rabbit reticulocyte lysates and in vivo, we considered it important to gain an understanding of HAV IRES function in a biologically relevant in vivo system. In order to study HAV translation in HAV-permissive cells, we constructed a cell line, BT7-H, which constitutively expresses bacteriophage T7 RNA polymerase. These cells are derived from continuous African green monkey kidney (B-SC-1) cells and remain permissive for HAV. The T7 polymerase supports transcription from transfected plasmid DNA which contains the T7 promoter. T7 transcripts are not capped in this cell line, and thus T7-producing cells should be useful for studying IRES function in vivo. HAV translation was evaluated in BT7-H cells transfected with a plasmid (pHAV-CAT1) containing the reporter gene chloramphenicol acetyltransferase (CAT), fused in-frame with the HAV 5'NTR and placed downstream of the T7 promoter. pHAV-CAT1 contains the 5'NTR sequence of the attenuated HM175/p16 virus, and has a T7 transcription terminator sequence placed immediately downstream of the CAT gene.

BT7-H cells were transfected with 5 μg of plasmid DNA using a liposome-mediated transfection system (Lipofectin, BRL). CAT expression was assessed at 48 hours post-transfection using a phase-extraction, liquid scintillation counting assay (Promega; pCAT Reporter Gene Systems). As shown in Figure 8A, the HAV IRES was far less active than the IRES of encephalomyocarditis virus (EMCV) in promoting expression of CAT. Average values for CAT activity many-fold less in cells transfected with pHAV-CAT1 than in those transfected with pEMCV-CAT (an analogous construct containing the EMCV IRES fused in-frame to CAT). This result was not due to differences in RNA transcription or stability, based on evaluation of CAT RNA levels by northern blot (data not shown).

Surprisingly, removal of much of the HAV IRES from pHAV-CAT1 increased expression of CAT about 3-fold (Figure 8A). Since a similar deletion in bicistronic constructs eliminated HAV IRES activity in rabbit reticulocyte lysates, it appeared likely that the translation of pΔ634-CAT does not depend on internal ribosomal entry, and perhaps occurred by ribosome scanning from the 5' end of the uncapped RNA. To determine whether this translation was dependent upon specific sequences in the residual HAV 5'NTR segment, a construct (pCN-CAT) was made in which the HAV IRES sequence was entirely replaced by a 53 base leader sequence derived from the pCAT-Control vector (Promega), which contains an AUG codon in excellent context (AAATGG) for initiation of CAT by scanning. CAT expression in cells transfected with pCN-CAT exceeded expression in cells transfected with pΔ634-CAT.

To verify that CAT translation in the BT7-H cells was cap-independent and to evaluate the effect of host-cell shutdown on HAV translation, expression from each of the CAT plasmids was assessed in the presence of the poliovirus protease, $2A^{pro}$ (Figure 8B). Poliovirus $2A^{pro}$ induces a proteolytic cleavage of the p220 subunit of the cap-binding complex eIF-4f, which is accompanied by shut-down of cap-dependent translation of cellular mRNAs. BT7-H cells were co-transfected with CAT plasmids and p2A-WT, which contains the T7 promoter and the EMCV IRES fused in-frame with the coding region for

poliovirus $2A^{pro}$ (a gift from R. Lloyd), and cell lysates were assayed for CAT activity as before. Since it has been suggested that the EMCV IRES itself may interfere with cap-dependent host-cell translation, possibly by competing for limiting quantities of factors required for translation, we also assessed expression from CAT plasmids following co-transfection with p2A-H20N (also provided by R. Lloyd), in which the poliovirus $2A^{pro}$ coding sequence has been altered to allow expression of a protease-inactive $2A^{pro}$. Expression of CAT from pEMCV-CAT was significantly enhanced in the presence of the active, but not inactive, poliovirus $2A^{pro}$ (data not shown). As an additional control, we evaluated the expression of CAT from pRSV-CAT (provided by S. Kenney), in which capped CAT transcripts are produced by nuclear (rather than cytoplasmic) transcription under the direction of the Rous sarcoma virus promoter. CAT expression from pRSV-CAT was virtually eliminated when cells were co-transfected with p2A-WT (Figure 8B), and reduced to a lesser extent in cells co-transfected with p2A-H20N. These results are consistent with the notion that expression of poliovirus $2A^{pro}$ abolishes cap-dependent translation, but also support the hypothesis that the EMCV IRES inhibits translation of capped transcripts by competing for necessary translation factors.

Like pEMCV-CAT, pΔ634-CAT and pCN-CAT expressed higher levels of CAT in the presence of poliovirus $2A^{pro}$ (Figure 8B). These results confirm that translation of transcripts from these plasmids is cap-independent. Moreover, these data indicate that an intact IRES is not essential for translation of certain uncapped transcripts in vivo, particularly in the absence of competing cap-dependent translation of cellular mRNAs. Surprisingly, we were unable to demonstrate enhanced expression of CAT from pHAV-1 in the presence of poliovirus $2A^{pro}$. This result suggests that HAV IRES efficiency may not be limited by competition with capped cellular mRNAs for translation initiation factors, and suggests indirectly that HAV replication is not impeded by the lack of host translational shut-off.

These experiments have demonstrated that the HAV IRES is far less active in promoting translation initiation in vivo than the structurally similar EMCV IRES. Transcripts in which most of the HAV 5'NTR was deleted, or replaced with a 5' leader which contained no HAV sequence, expressed significantly greater quantities of CAT than transcripts containing the entire HAV 5'NTR. The data suggest that translation of uncapped transcripts having reduced secondary structure at the 5' end (e.g., p634-CAT and pCN-CAT) occurs by scanning of ribosomes from the 5' end and does not require an intact IRES. However, it appears that stable secondary structures present in the HAV sequence upstream of base 634 are inhibitory to translation by this mechanism, requiring that translation initiation occur by internal ribosomal entry. It is likely that the low activity of the HAV IRES in vivo is at least partly responsible for the slow growth and noncytolytic replication cycle of HAV. If this is the case, mutations in the 5'NTR which enhance growth in cell culture would be expected to enhance IRES activity in relevant cell lines. This hypothesis is currently under investigation.

7. Cellular proteins which bind to the 5'NTR of HM175/p16 virus

Work described in the Section 4 has shown that mutations in the 5'NTR of HM175/p16 virus play an important role in determining the ability of this virus to replicate in certain cell types. The attenuation phenotype of this virus is likely to be related to the same mutations which define its ability to replicate in African green monkey kidney cells (such as BS-C-1 cells). We consider this likely because attenuation of this virus was positively selected for during its adaptation to growth in this cell type, and because attenuation of cell culture-adapted hepatitis A viruses appears to be due to an altered virus host range. Mutations in the 5'NTR which enhance growth in cell culture are likely to function by altering the affinity of the viral RNA for specific cellular factors which interact

with the 5'NTR, and which are capable of influencing viral replication. Cellular translation initiation factors are good candidates for such proteins, although it is possible that cell proteins also play roles in replication of the viral RNA or assembly of the virion (both of which are also likely functions of the 5'NTR).

To define potential interactions between the HAV 5'NTR RNA and cellular factors involved in translation, we characterized the binding of ribosome-associated proteins present in several cell types to synthetic RNAs representing segments of the 5'NTR using a UV cross-linking/label transfer assay. Five major proteins (p30, p30.5, p39, p57 and p110) were identified (Figure 9). p30 and p39 were present in ribosomal salt washes prepared only from HAV-permissive BS-C-1 and FRhK-4 cells, while p57 was found only in HeLa cells and rabbit reticulocyte lysates. p30.5 and p110 were present in all cell types. Both p30 and p39 bound to multiple sites within the 5'NTR. Efficient transfer of label to p30 occurred with minimal RNA probes representing nts 96-155, 151-354, and to a much lesser extent 634-744, while label transfer to p39 occurred with probes representing nts 96-155 and 634-744. All of these probes represent regions of the 5'NTR which are rich in pyrimidines. Competitive inhibition studies indicated that both p30 and p39 bound with greater affinity to sites in the 5' half of the NTR (a probe representing nts 1-354), than to the more 3' site (probe 634-744). Binding of p39 (but not p30 or p110) to the probe representing nts 96-155 was inhibited in the presence of an equal amount of proteins derived from HeLa cells, suggesting that p39 shares binding site specificity with one or more HeLa cell proteins. These results demonstrate that ribosome-associated proteins which bind to the 5'NTR of HAV vary substantially among different mammalian cell types, possibly accounting for differences in the extent to which individual cell types support growth of the virus. We suspect that mutations in the 5'NTR which enhance the growth of HAV in certain cell types may reflect specific adaptive responses to these or other proteins.

Work at present is directed at determining the nature of the p39 protein. Preliminary results indicate that this protein shares binding specificity with polypyrimidine tract binding (PTB) protein, a ubiquitous cellular protein which may play a role in nuclear spicing reactions and which has been shown to bind specifically to the 5'NTR of EMCV (E. Wimmer, personal communication). We have recently shown that a recombinant PTB fusion protein (gift of M. Garcia-Blanco) competes with BS-C-1 cell-derived p39 for binding to selected HAV RNA probes.

8. Mutations associated with cytopathic HAV

The HM175/18f virus is a rapidly replicating, cytopathic variant of HM175 virus (RR/CPE$^+$ phenotype) which was recovered from persistently infected cells (see Reports Nos. 1 and 2) (Lemon et al., 1991). We reported in Annual Report No. 3 that we had constructed a series of recombinant full-length infectious cDNA clones in which we replaced the 5'NTR, P2 and 3'NTR regions of the infectious pHAV/7 clone with cDNA amplified from corresponding regions of HM175/18f virion RNA by polymerase chain reaction. The validity of these constructions has been confirmed by DNA sequence analysis. Transfection of FRhK-4 cells with RNA transcribed from these constructs has shown that mutations in the P2 region of HM175/18f confer a rapid replication (RR+) phenotype on the HAV/7 virus (generating large foci in 7 days compared with 14 days in transfection/radioimmunofocus assays) (see Report No. 3). These results were subsequently confirmed in BS-C-1 cells using virus rescued from the original transfections. Surprisingly, there was no apparent enhancement of the growth of pHAV/7 (HAV/7) recombinants in either cell type with the additional inclusion of the 5'NTR of HM175/18f virus. Given the mutations in HM175/18f defined by us previously (see Annual Report No. 2), these results suggest an important role for the 2C gene of HM175/18f virus in controlling the enhanced growth of this virus in cell culture. However, radioimmunofoci

generated by HAV/7 recombinants containing the 5'NTR, P2 and 3'NTR sequences of HM175/18f, while much larger than foci of the parental HAV/7 virus, are significantly smaller than foci generated by HM175/18f virus itself. This suggests that additional mutations in HM175/18f virus, outside of the 5'NTR, P2 and 3'NTR regions, contribute significantly to its growth properties. We are currently constructing additional chimeric viruses to define in greater detail the role played by individual P2 proteins, as well as other regions of the HM175/18f genome, in determining the RR/CPE$^+$ phenotype. These studies are important because they are providing information concerning the genetic determinants of rapid growth of HAV in cell cultures. This information may prove useful in designing candidate vaccine viruses which replicate more efficiently in cell culture.

CONCLUSIONS

During the past year, we have largely completed analysis of a linear antigenic determinant on the surface of the HAV particle. We have confirmed that a neutralizing monoclonal antibody (H7-C27) specifically binds synthetic peptides representing domains which are either near to or located at the carboxy terminus of VP1. To date, these peptides have not proven capable of inducing neutralizing antibodies to HAV, but additional peptides are being synthesized based on the results of PEPSCAN studies, and these additional peptides will be tested for both antigenicity and immunogenicity.

We have developed a very sensitive and specific immunoprecipitation method for detection of antibodies to HAV. This radioimmunoprecipitation assay is substantially more sensitive than much more labor-intensive virus neutralization assays. Preliminary characterization of this antibody test suggests that it will be very useful in monitoring the immune response to hepatitis A vaccines, as it is capable of detecting low but protective levels of antibodies following a single dose of the Merck inactivated hepatitis A vaccine. Further studies examining the antibody response to this vaccine are currently in progress.

We have defined the attenuation phenotype of the cell culture-adapted HM175/p16 virus, and have shown that mutations within the 5' nontranslated region (5'NTR) of its genome contribute to its ability to grow in cell culture in a very cell type-specific function. These findings have prompted a detailed examination of the structure and function of the 5'NTR, including the characterization of cellular proteins which bind specifically to this region of the viral RNA. These studies have shown that the 5'NTR of HAV contains an internal ribosomal entry site (IRES) similar to that of other picornaviruses, but that the IRES of HAV has very weak activity in directing translation in vivo. This weak translational activity is likely to be important in determining the generally slow and noncytopathic replication cycle of HAV. These studies promise a better understanding of the basic mechanisms underlying adaptation of HAV to growth in cell culture. Because such mechanisms are likely to be closely related to attenuation of cell culture-adapted HAVs, this knowledge should prove useful in refining current attenuated vaccine candidates, or in designing novel candidate attenuated vaccine strains.

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APPENDIX

Table 1. H7-C27 EPITOPE: REPLACEMENT SYNTHESIS

Octapeptide	O.D.
260 265 Y F P R A P L N	0.315
T F P R A P L N	0.201
Y F G R A P L N	0.527
Y F P R A G L N	0.110
Y F G R A G L N	0.113
P R A P L N S N	0.662
P R A G L N S N	0.096
G R A G L N S N	0.114

Note: The O.D. shown represents detection of the indicated octapeptide with the H7-C27 murine monoclonal antibody.

Table 2. HAV VP1 SYNTHETIC PEPTIDES

Peptide	Peptide Sequence
4918	260 EFYFPRAPLNSNA-GGC- [KLH]
4919	[KLH] -GG-EFYFPRAPLNSNA 260
4920	260 270 280 EFYFPRAPLNSNAMLSTESMMSRIAAGD-C- [KLH]

Table 3. Anti-VP1 Peptide Antisera

Peptide	Serum	Species*	Octapeptide ELISA Spanning VP1 Residues	Neut. anti-HAV (RIFA)
4918	gpA1	gp	257-270	<1:8
	gpA2	gp	258-268	<1:8
	rb3314	rab	256-269	<1:8
	rb3321	rab	257-272	<1:8
4919	gpA3	gp	258-270	<1:8
	gpA4	gp	not done	<1:8
	rb3290	rab	260-268	<1:8
	rb3353	rab	non-reactive	<1:8
4920	gpA5	gp	256-268, 273-284	<1:8
	gpA6	gp	254-268, 273-284	<1:8
	rb3286	rab	257-267, 273-282	<1:8
	rb3279	rab	non-reactive	<1:8

*gp = guinea pig; rab = rabbit

Table 4. Detection of anti-HAV in Children Immunized with the Merck Inactivated HAV Vaccine

(25 antigen unit doses given at months 1 and 6)

Patient	Month	Antibody to HAV		
		RIFIT	RIP >30%*	RIP >50%*
27	0	< 1:8	< 1:8	< 1:8
	1	< 1:8	≥ 1:800	1:80
	6	< 1:8	1:80	1:8
	7	≥ 1:800	≥ 1:800	≥ 1:800
41	0	< 1:8	< 1:8	< 1:8
	1	< 1:8	1:80	1:80
	6	1:8	≥ 1:800	≥ 1:800
	7	≥ 1:800	≥ 1:800	≥ 1:800
71	0	< 1:8	< 1:8	< 1:8
	1	1:8	≥ 1:800	≥ 1:800
	6	1:8	≥ 1:800	≥ 1:800
	7	≥ 1:800	≥ 1:800	≥ 1:800
91	0	< 1:8	< 1:8	< 1:8
	1	1:8	1:80	< 1:8
	6	< 1:8	1:80	1:80
	7	≥ 1:800	≥ 1:800	≥ 1:800
101	0	< 1:8	< 1:8	< 1:8
	1	< 1:8	≥ 1:800	1:80
	6	< 1:8	< 1:8	< 1:8
	7	≥ 1:800	≥ 1:800	≥ 1:800

*Radioimmunoprecipitation assay, >30% and >50% precipitation endpoints

Table 5. Response of Aotus nancymai to Intravenous Inoculation with Wild-Type, HM175/p16 and HM175/S18 Hepatitis A Viruses

Virus*	Inoculum Size	Seroconversion† Frequency	Seroconversion† Mean Days PI	ALT Elevation** Frequency	ALT Elevation** Mean Maximum	Radioimmunoassay Detection of Fecal HAV†† Frequency	Radioimmunoassay Detection of Fecal HAV†† Mean Max S/N
HM175 Human feces n=6	?	5/6	<28	5/6	8.5	5/6	73.2
HM175/p16 Passage = 16 n=4	$10^{4.5}$ rfu	3/4	103.2	1/4	3.2	0/2	-
HM175/S18 Passage = 22 n=6	$10^{3.5}$ rfu	6/6	26.6	6/6	6.5	6/6	14.6

*Passage indicates total number of cell culture passages. n = number of animals inoculated with each virus. Wild-type infections were described by LeDuc et al. (1983) amd HM175/S18 infections by Lemon et al. (1990).

†Seroconversion by competitive inhibition immunoassay; mean number of days after inoculation (PI) when antibodies to HAV were first documented. Animal 1022 (present study), which developed antibodies between 96 and 307 days after inoculation, was considered to have seroconverted on day 202. The time of seroconversion was not precisely determined in the wild-type virus challenge, but 3 of 5 seroconverters were anti-HAV positive by 28 days after inoculation.

**Frequency = proportion of inoculated animals which developed ALT > 2.5 x baseline (individually determined for each animal); Maximum = mean maximum-fold elevation, for animals with elevations significantly above baseline.

††Fecal HAV antigen was measured by radioimmunoassay only in animals which developed antibodies to HAV within 96 days of inoculation (see Table 2). Radioimmunoassays in different challenge experiments were not run simultaneously. "Max S/N" = mean maximum signal/noise ratio.

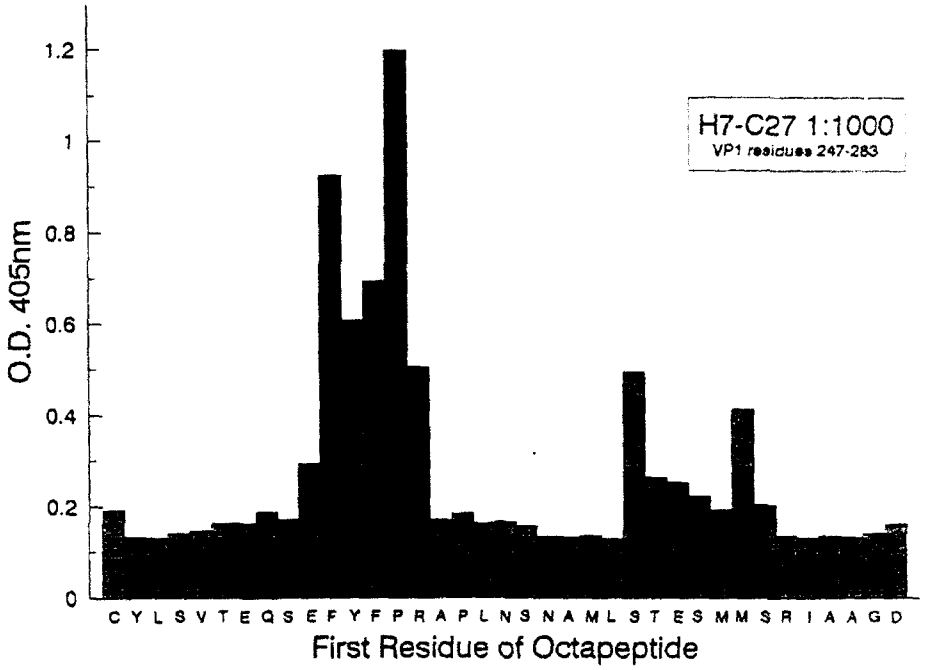

Figure 1. PEPSCAN analysis of HAV VP1 residues 247-283 (octapeptide scan): H7-C27 murine monoclonal antibody diluted 1:1000.

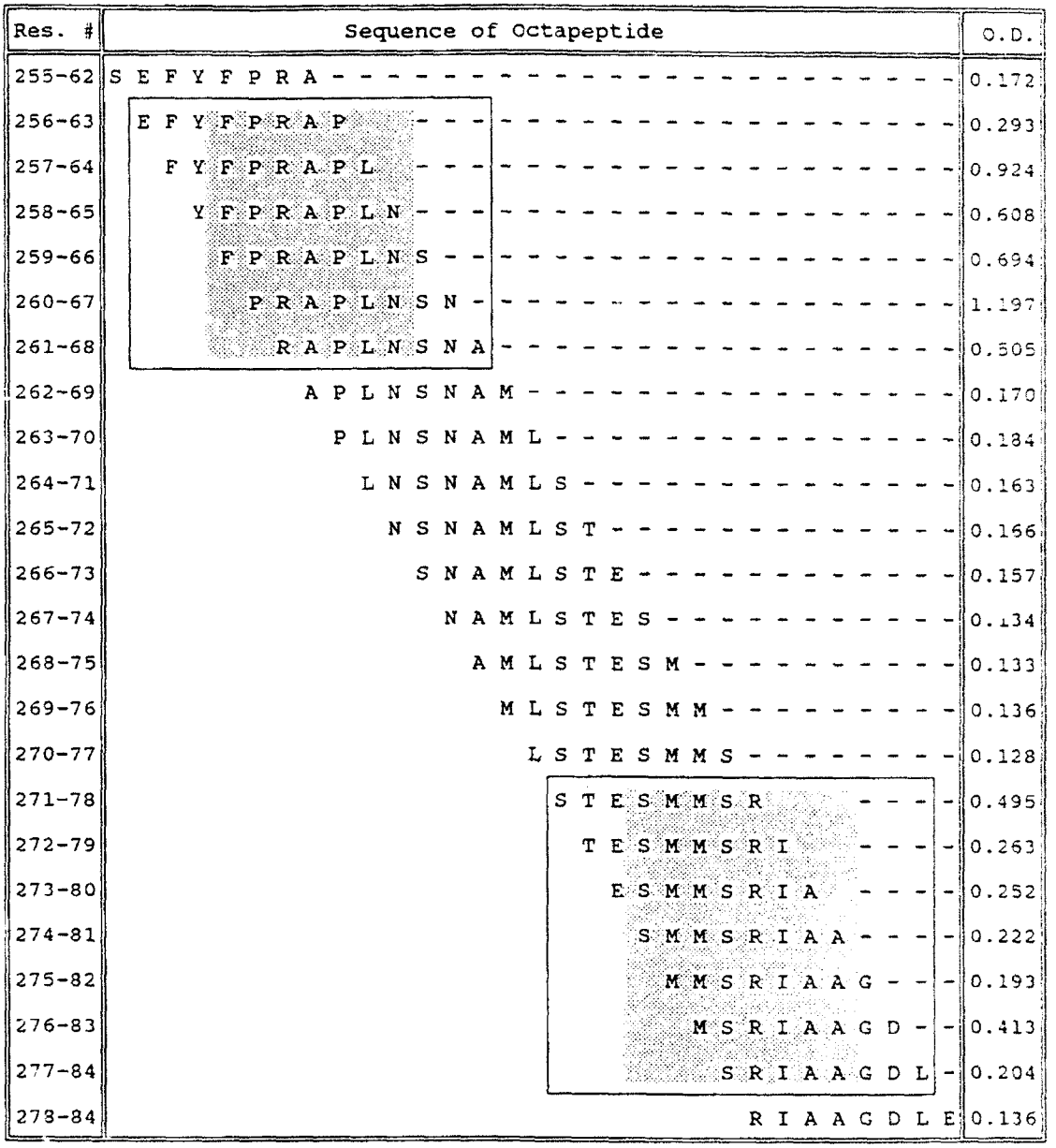

Figure 2. Detailed analysis of a discontinuous HAV neutralization epitope detected by an octapeptide scan with murine monoclonal antibody H7-C27. Boxed peptides generated O.D. values greater than baseline; shaded residues represent core regions of the epitope, based on predictions that the minimal size of an antigenic peptide is 5 residues.

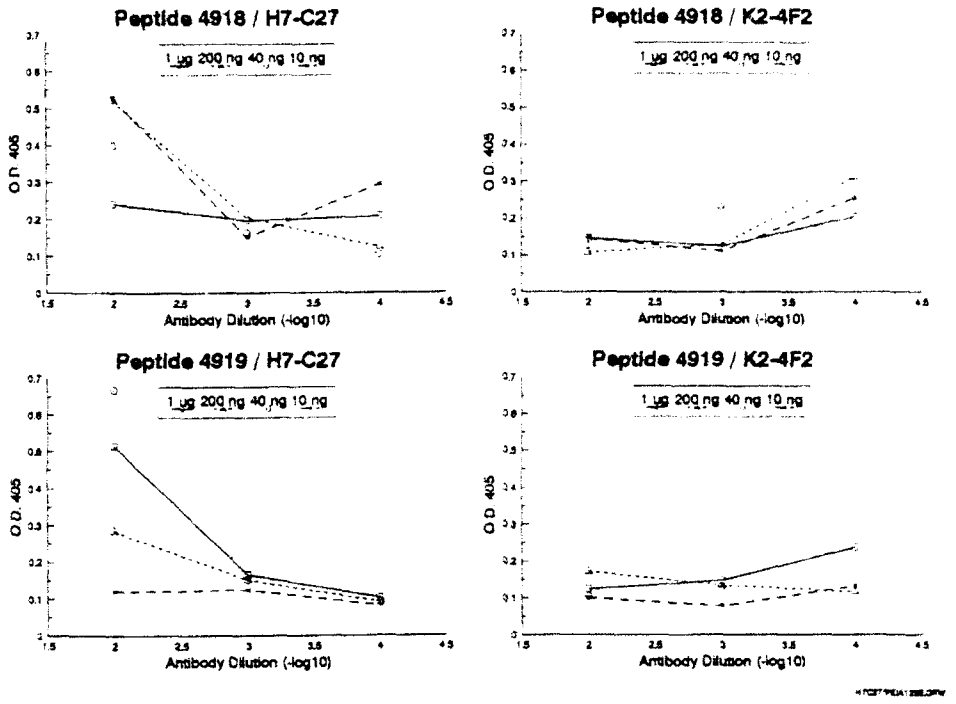

Figure 3. Solid-phase microplate peptide ELISAs utilizing peptides 4918 and 4919 and two neutralizing murine monoclonal antibodies to HAV: H7-C27 and K2-4F2.

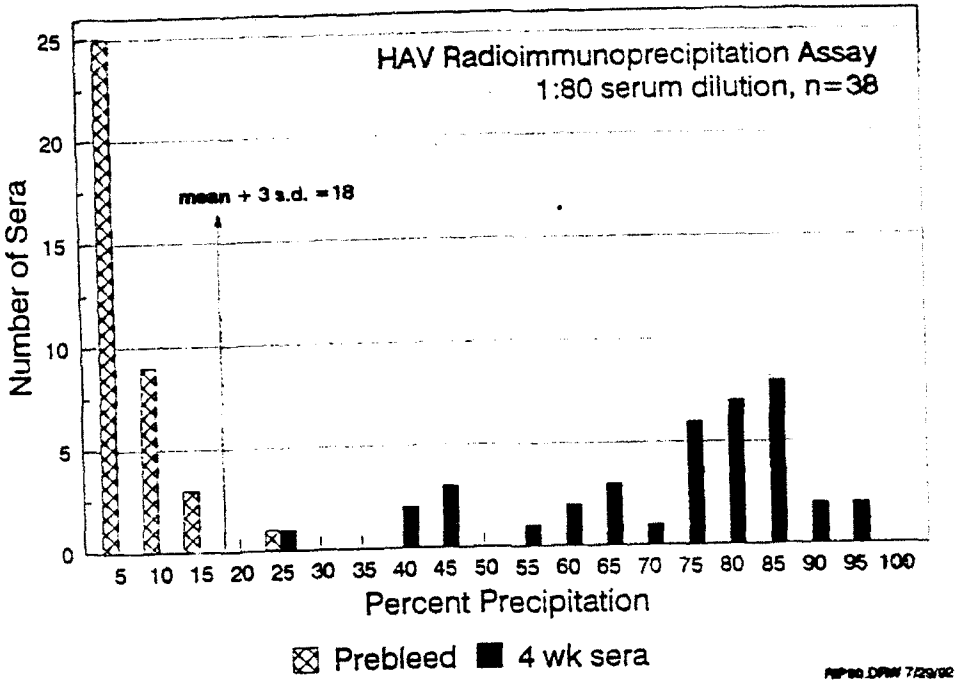

Figure 4. Detection of anti-HAV by [^{3}H]-HAV immunoprecipitation in sera collected from children before and after a single dose of inactivated HAV vaccine. Sera were tested at a 1:80 dilution. The arrow indicates the mean precipitation, plus 3 s.d., of results obtained with prebleeds.

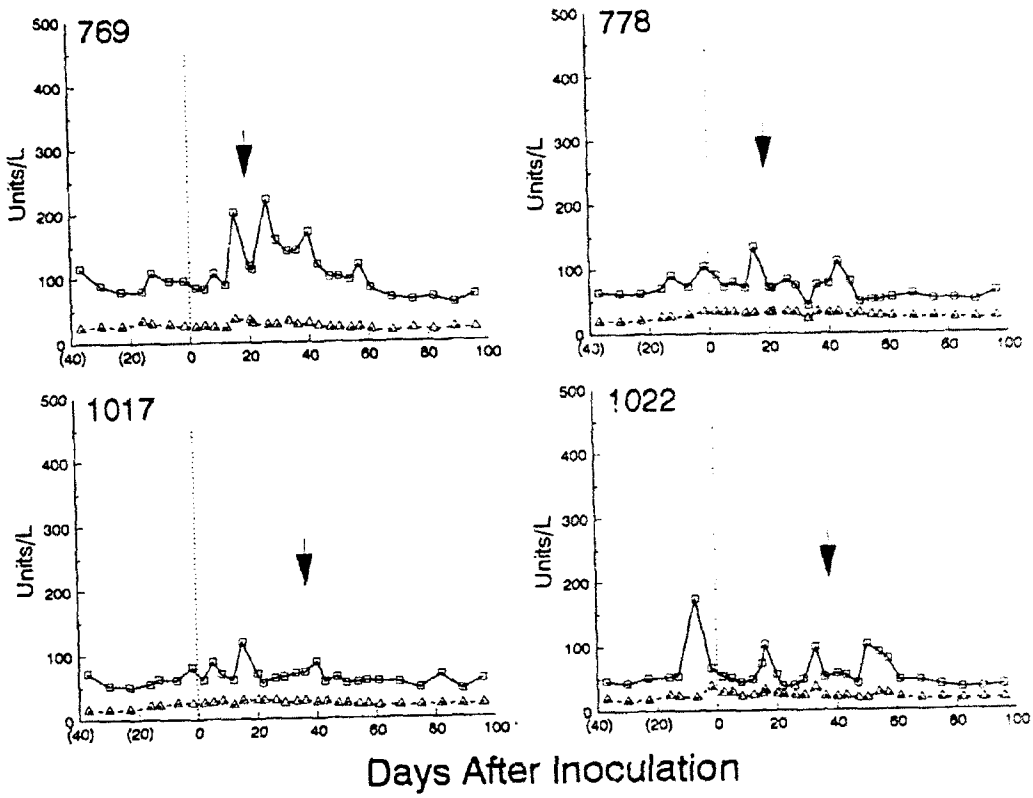

Figure 5. Serum enzyme activities (ALT and GGT) in 4 New World owl monkeys before and after intravenous inoculation with attenuated HM175/p16 virus.

(ALT = open squares; GGT = open triangles)

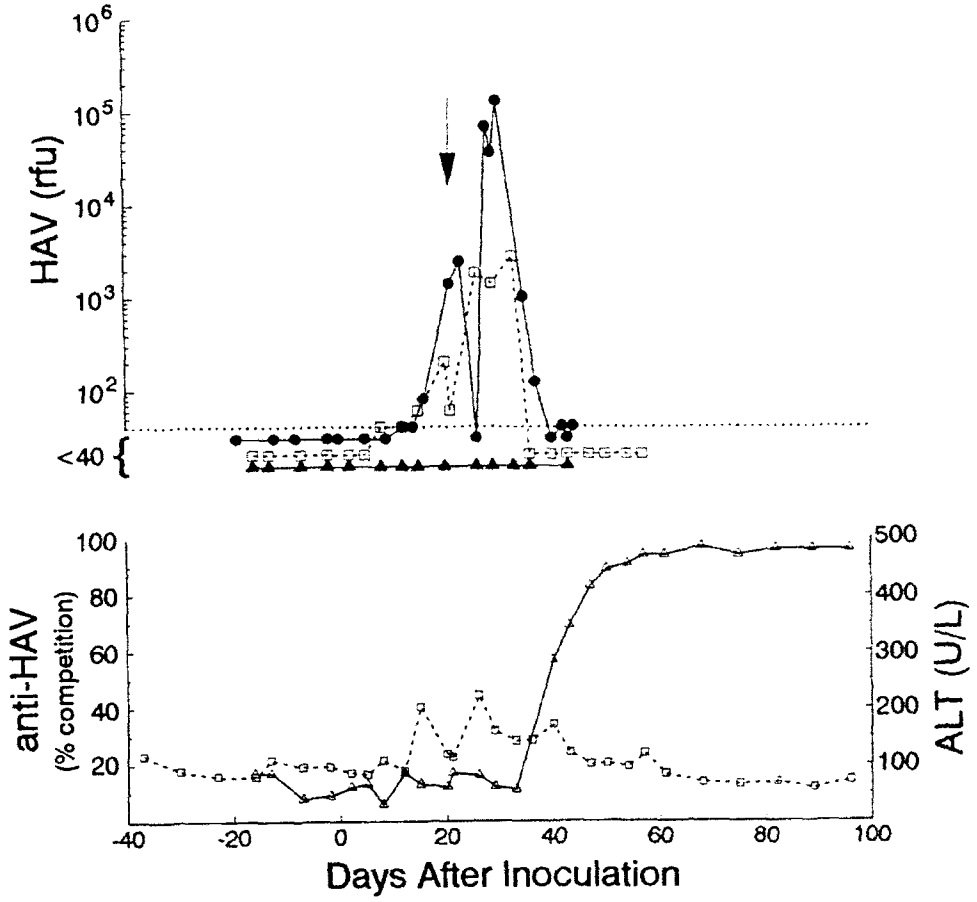

Figure 6. Virologic and serologic events following challenge of animal 769 with HM175/p16 virus. The top panel depicts the titer of virus present in fecal suspensions (solid circles, radioimmunofocus-forming units (rfu)/gm feces) and serum specimens (open squares, rfu/ml) collected following i.v. inoculation of virus. No virus was isolated from specimens shown at points below the dotted horizontal line (<40 rfu/gm feces, or /ml serum). Virus was not isolated from any throat swab specimen (solid triangles). The arrow indicates timing of the second open liver biopsy. The bottom panel depicts serum ALT activities (open squares, units/L) and anti-HAV response determined by competitive-inhibition radioimmunoassay (open triangles, percent competition with [^{125}I]-labeled human polyclonal anti-HAV IgG for binding to immobilized HAV) following virus challenge. Greater than 50% competition in the radioimmunoassay is considered indicative of the presence of specific anti-HAV antibodies.

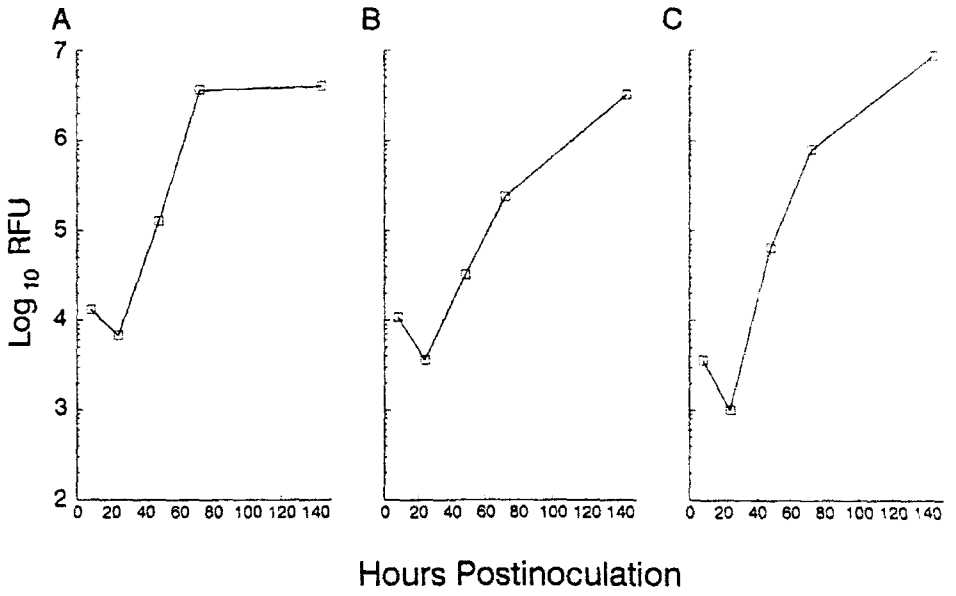

Figure 7. Intracellular virus accumulation measured under approximate one-step growth conditions in BS-C-1 cells infected with virus rescued from: A, pA/7-5'p16[25-532] (HAV/7 virus with 5'NTR mutations at 152 and 203-7); B, pA/7-5'wt (HAV/7 virus with 5'NTR of wild-type virus); C, pG3HAV/7 (HAV/7 virus).

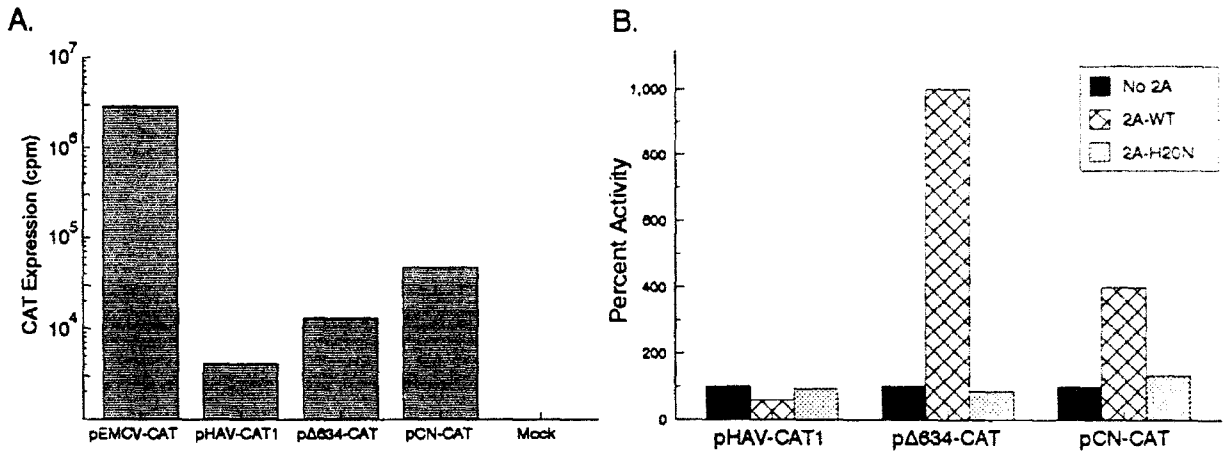

Figure 8. (A) Expression of CAT in BT7-H cells transfected with contructs containing the CAT gene under control of the 5'NTR of HAV or EMCV. See text for details. (B) CAT expression in BT7-H cells transfected with HAV-CAT constructs and co-transfected with plasmids containing the poliovirus 2A gene under control of the EMCV IRES. See text for details.

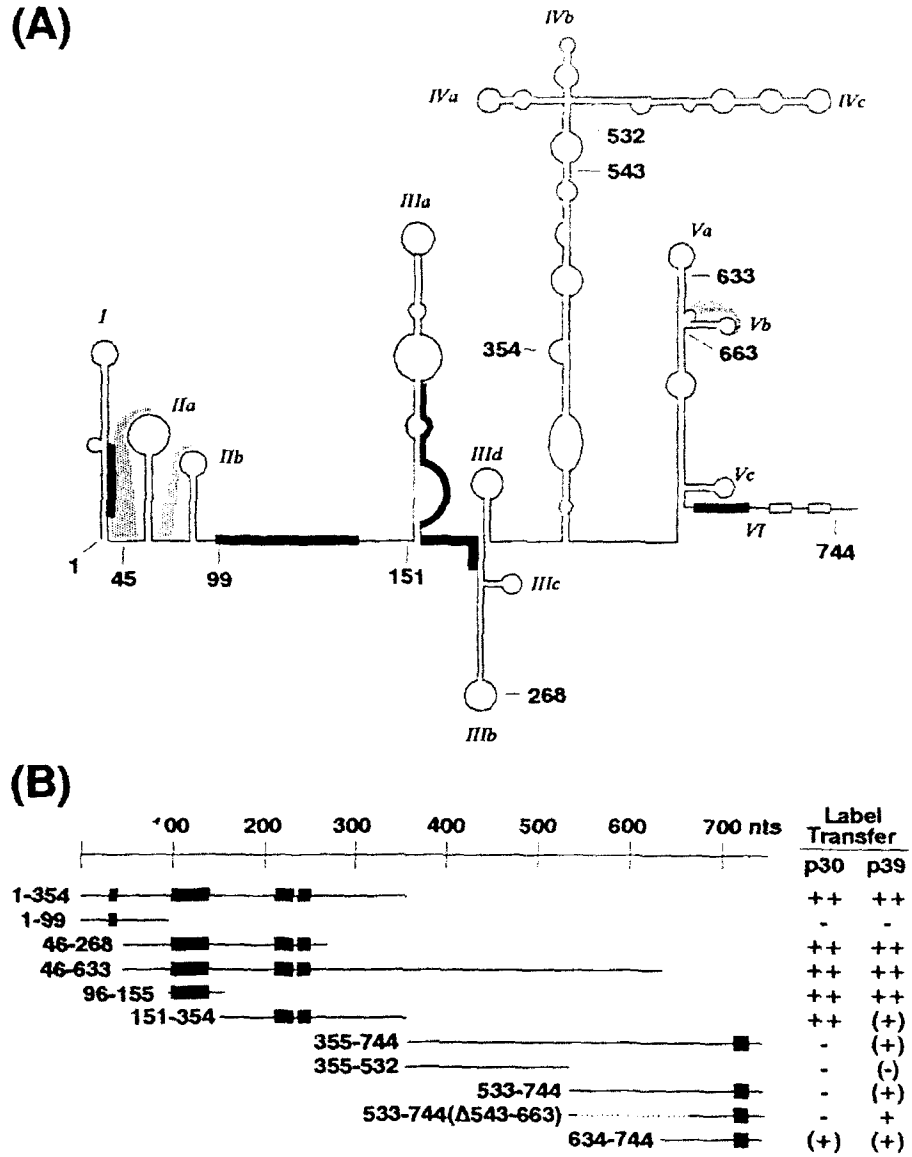

Figure 9. RNA probes synthesized for UV cross-linking experiments. (A) Schematic representation of the proposed secondary structure of the HAV 5' NTR (not to scale) (see Brown et al., 1991). Major structural domains are indicated by roman numerals I-VI, while individual stem-loops within each domain are designated by lower case letters (e.g., IIIa). The two potential initiation codons (AUG-11 and AUG-12) are shown as open boxes, while pyrimidine-rich tracts (defined as continuous strings of at least 8 pyrimidines, and contiguous pyrimidines including no more than a single purine) are shown as thick lines. Stippled regions indicate potential base pairing which may result in RNA pseudoknots. The positions of restriction sites used to generate plasmid constructs and RNA substrates are numbered (wild-type numbering). (B) Linear representation of RNA probes. Map units (nucleotide positions) are shown at the top. Probes are designated at the left according to their 5' and 3' limits, while the extent of label transfer to the major BS-C-1 proteins (p30 and p39) in UV cross-linking studies is indicated at the right. The dashed line indicates a deletion, while the thick lines indicate the location of pyrimidine-rich tracts defined as above.